Developing CAR-T Therapy for Treating B Cell Malignancies

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Abstract: B cell lymphoma is a type hematopoietic malignancy with an average incidence rate of 4.3%. While B cell lymphoma is not as deadly as other solid tumors, aggressive lymphomas such as the diffuse large B cell lymphoma (DLBCL) can be fatal due to its fast-spreading characteristic and high metastatic ability. To more effectively target B-cell lymphomas, scientists have recently created the chimeric antigen receptor (CAR) that can actively detect the CD19 antigens secreted by cancer cells and directly activate T cells without binding to the major histocompatibility complex (MHC). However, over time, many cancer cells have also developed several mechanisms to escape the detection of T cells and to inhibit their function, which can significantly hamper the overall efficacy of traditional CAR-T therapy. Furthermore, traditional CAR-T therapies may also cause severe side effects, such as the cytokine release syndrome (CRS) caused by an overproduction of proinflammatory cytokines. In this study, we examined six current research articles that address these immune escape mechanisms as well as the side effects caused by traditional CAR-T therapies. We propose an experimental CAR-T therapy that combines the major findings from this primary research, which, if proven feasible, can substantially improve the overall efficacy of CAR cancer immunotherapy while significantly reducing damage caused by side effects.

1 INTRODUCTION

B cell lymphoma is a hematopoietic malignancy characterized by the proliferation of abnormal B lymphocytes (Swiner, 2020). Most cases of B cell lymphoma belong to the category of non-Hodgkin lymphomas (NHL) such as the fast growth DLBCL and the indolent chronic lymphocytic leukemia (CLL). CLL generally grows much slower than aggressive types, but they are also less curable with standard treatments and, if left untreated, can potentially grow into a more aggressive form of cancer. Additionally, even though 40-50% of all patients with DLBCL can achieve complete remission after therapy, 30-40% of patients relapse within a short time and 10% develop refractory DLBCL, a type of cancer that does not respond to any type of treatment. Patients with relapsed/refractory DLBCL are less responsive towards conventional cancer therapies, and even if receiving second treatments with higher doses of chemotherapy and stem cell transplants, r/r DLBCL patients have a 1-year survival rate of only 28% (Raut, 2014). These characteristics make B cell lymphoma more dangerous compared to other cancers. As a result, there exists a dire need for new cancer therapies that specifically target B cell lymphoma.

Current cancer treatment options include surgery, chemotherapy, and radiation therapy, as well as the latest techniques such as the minimally invasive interventional radiology and immunotherapy. However, because chemotherapies may cause serious side effects such as non-specific cytotoxicity and generalized immune suppression, scientists have recently been investigating different immunotherapies that utilize the immune system itself to offer long-term remission through immunological memory. Among different cancer immunotherapies, CAR-T therapy that involves the

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manipulation of T lymphocytes in the adaptive immune system has shown significantly greater efficacy against B cell lymphoma in multiple research studies and clinical trials.

1.1 CAR-T Therapy

The activation process of T lymphocytes involves the interaction between the T cell receptor (TCR) and specific antigens presented by the MHC as well as several co-stimulation factors such as CD28, CD137, and OX40. However, to evade T-cell detection tumor cells have developed several immune inhibitory functions, such as upregulating inhibitory molecules as well as reducing MHC expression (Yilmaz, 2020).

CAR-T therapy addresses these limitations by creating T cells that function independently of MHC molecules (Graham, 2018). CARs are split into three domains: ectodomains, a transmembrane domain, and an intracellular domain of CD3ζ for signal transduction (Figure 1). The ectodomain is the most vital and different from traditional TCRs; it consists of a signal peptide and the antigen recognition domain of the single-chain Fragment variant (ScFv) derived from the F_{ab} region of antibodies fused by a linker.

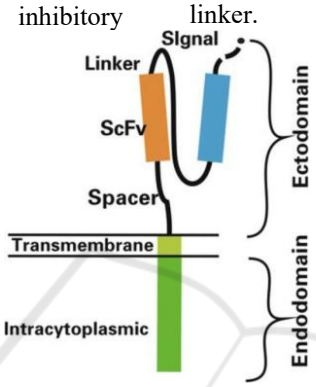

Figure 1: Structure of a Chimeric Antigen Receptor.

The Ectodomain consists of the linker, spacer, ScFV head, and signal joint. The single-chain variable fragment (ScFv) is responsible for the specific antigen recognition from CAR cells. Composed of the Fab regions of light and heavy chains of an immunoglobulin, the ScFV is connected to the CAR via a short linker peptide. The spacer, also known as the hinge region, connects the antigen recognition region to the surface of the membrane. It enhances ScFV flexibility and promotes the binding of recognition regions to target cells (Guedan, 2019).

Advantages of CAR-T arises from the fact that they are MHC-independent, which allows it to recognize any type of surface antigen, including carbohydrates and lipids. Additionally, as memory T cells remain in circulation long-term, the benefits of CAR-T therapy can last for several years. CAR-T therapies have little time for treatment, as it consists of a single injection after which the patient can be released after two weeks. Because the treatment itself is not as aggressive as chemotherapy and radiation, patients tend to have a much more rapid recovery. Additionally, CAR-T can be a final resort for patients who do not qualify for stem cell transplantation or suffer from multiple relapses.

1.2 Side Effects of CAR-T Therapy

To date, CAR-T cells have shown remarkable antitumor activity in patients because of the long-lasting remissions in hematologic malignancies that are not responding to standard therapies. For example, the CAR-T therapy Axicabtagene Ciloleucel can reach a tumor objective response rate of 82% and a complete response rate of 54% (Levine, 2016). However, CAR-T therapies also have serious limitations, such as the CRS. CRS is a severe side effect of CAR-T therapies, in which the signalling mechanism involved can provoke secretion of cytokine and activation of macrophages (Neelapu, 2017). As a result, while investigating mechanisms to decrease this side effect in CAR-T therapies, researchers have also started to incorporate natural killer (NK) cells and nanobodies in CAR-Therapies.

2 PRIMARY RESEARCH STUDIES

To resolve different immune escape mechanisms developed by tumor cells, we first examined two papers, which introduced the idea of engineering

CAR-T cells to make CAR receptors actively secrete anti-PD-1 antibody and CD40 ligand (CD40L). Consequently, we evaluate the efficacy of the IL-6 binding protein in reducing the CRS discussed in the third study. To maximize the overall effectiveness of CAR-T therapy, we further examined the effector function of different subsets of T cells discussed in the fourth paper.

2.1 Engineered CAR-T Cells with Self-Secreting Anti-PD1 Antibodies Have Shown Optimistic Results in Suppressing PD-1 Inhibitory Receptors in the Tumor Microenvironment (TME) of CD-19 Expressing Tumors

CAR-T treatments' long-term efficacy has been impeded by the upregulation of several cell surface inhibitory molecules in the TME, such as cytotoxic T lymphocyte-associated protein (CTLA-4) and programmed death-1 (PD-1). These inhibitory receptors can negatively regulate the proper activation of T cells by competing with the co-stimulator CD28 for the B7 ligand. Without proper activation, the overall immune response generated by the adaptive immune system will be largely hampered (Li, 2017). Among different inhibitory molecules, the upregulation of PD-1 in CAR-T cells can cause not only hypofunction of CAR-T cells but also dysfunction of tumor infiltrate cells (TLC) following specific antigen stimulation. As a result, in this study, scientists aimed to combine the CAR-T therapy with the PD-1 blockade mechanism to overcome the inhibitory effect of PD-1. They conducted the experiment using lung cancer line NCI-H292 and designed a special CAR-T cell, CAR19.alphaPD1, targeting CD-19 antigen by inserting a gene fragment that can express the ScFv from anti-PD1 antibody using retroviral vector. To test whether CAR19.alphaPD1 can reduce the immune inhibitory effect caused by PD-1, researchers performed a competitive binding and blocking assay on both CAR-T and CAR19.alphaPD1 cells. In this experiment, T cells' activity after being stimulated by anti-CD-3 antibodies was measured by intracellular IFN gamma in the assay. According to the results, after the recombinant human PD-L1 ligand were applied to each well in the assay, the IFN gamma level significantly, but this reduction of IFN gamma count was quickly reversed after the addition of CAR19.alphaPD1, indicating a successful blockade of PD1 receptor by the self-secreted anti-PD-1 antibody. In addition, although PD-1 was upregulated in both CAR-T and CAR19.alphaPD1 following antigen stimulation, researchers in this study have also discovered that expression of PD-1 was significantly lower in CAR19.alphaPD1 T cells compared to parental T cells, which can further reduce the inhibitory effects caused by PD-1 in the TME. Furthermore, in multiple other tests on CAR19.alphaPD1's ability to enhance antigen-specific immune response, antitumor reactivity, T cell proliferation, and T cell effector function, CAR19.alphaPD1 in these tests all showed improved efficacy compared to the control.

Based on the results, CAR19.alphaPD1 has proven to be more effective in targeting H292-CD19 bearing tumors as well as reducing the immune inhibitory signal in the TME compared to parental CAR-T cells. Overall, CAR19.alphaPD1 can be considered as an effective way to target CD19-expressing tumors in future cancer immunotherapies.

2.2 Engineered CAR-T Cells with the Ability to Secrete CD40L Can Effectively Prevent Tumor Immune Escape Caused by Antigen Loss

Tumor immune escape can occur via antigen loss so that CAR-T cells lose their targets. To overcome these negative impacts, CAR-T cells are engineered to constitutively express CD40L to increase the activation of the CD40/CD40L pathways in B cell lymphoma. CD40 receptors are located on the surface of abnormal B cells in B lymphoma. By transiently activating CD40, CD40L can direct anti-proliferation and apoptosis signals to cancer cells, thus effectively reducing the outgrowth of B lymphoma (Odorizzi, 2012).

In addition, the CD40/CD40L pathway is highly utilized for the activation of APCs, such as dendritic cells (DC). The recruited APCs can further initiate antitumor T cell responses by activating $CD4^+$ and $CD8^+$ as well as secreting IL-12, which functions by inhibiting the suppressive function of immune-inhibitory macrophages, enhancing the antitumor response of CAR-T cells, and recruit other non-CAR-T cells at the same time (Elgueta, 2009).

Because the $CD19^-$ tumor can escape lysis by traditional anti-CD19 CAR-T cells used in this study (m1928z), the effectiveness of engineered CAR-T cells with CD40L (m1928z-CD40L) on $CD19^-$ tumor cells needs to be investigated. In this research study, scientists modified the $CD19^+$ A20 lymphoma cell line with the green fluorescent protein (GFP) and co-

cultured $CD19^+$ GFP^+ A20 cells with both m1928z CAR-T cells and m1928z-CD40. The results showed that by day 21, the outgrowth of $CD19^-$ tumor cells can be successfully eliminated by m1928z-CD40L compared to m1928z, indicating that m1928z-CD40L is more effective in detecting antigen-negative tumor cells in the long term through the increased activation of the CD40/CD40L pathway. (Elgueta, 2009).

2.3 CAR–T Cells with Mbail6 Have Complete Antitumor Activity and Neutralize Macrophage-Derived IL-6, Which Could Prevent CRS

The CRS has been one of the most severe side effects of traditional CAR-T therapy since the development of the first generation of CAR-T cells. Pathophysiologically, CRS is mediated by proinflammatory cytokine interleukin-6 (IL-6) mainly secreted by activated macrophages (Kuhn, 2019). Patients with CRS experience acute systemic inflammatory responses characterized by fever, fatigue, and headaches, which can be life-threatening in some cases. Currently, the IL-6 receptor inhibitor tocilizumab has been approved by FDA to treat CRS, yet its effect is not stable. In this study, researchers use a non-signaling membrane-bound IL-6 receptor (mbaIL6) that possesses anti-CRS activity while maintaining CAR-T cells' complete antitumor capacity. Compared with the control groups, which are Jurkat cells with only GFP expression, mbaIL6-expressing Jurkat cells showed a significant reduction of IL-6 concentration in cell culture. The neutralization of IL-6 by mbaIL6 is further tested using the U937 cell line, in which stimulation is dependent on IL-6-mediated STAT3 phosphorylation. When cocultured with mbaIL6-expressing Jurkat cells, STAT phosphorylation reaction was significantly reduced compared to the control group. These results imply a successful neutralization of IL-6 by mbaIL6, which is critical in reducing CRS in CAR-T therapies.

Consequently, researchers engineered traditional CAR-T cells using a bicistronic MSCV vector containing genes encoding mbaIL6 and anti–CD19-41BB-CD3z CAR. The mabIL6 expression in peripheral blood T lymphocytes was proved to have no interference with the immunophenotype of T cells. Further experiments have also shown that the expression of mabIL6 on anti-CD-19 CAR-T cells does not affect their normal proliferation rate, suggesting that a combination of anti-CD-19 CAR and mabIL6 could be a frontline treatment for B-lymphoid malignancies and multiple myeloma (Neelapu, 2017).

2.4 Using a Defined Subset of CD8+ TCM and CD4+ TN Cells in a 1:1 Ratio Has Synergistic Antitumor Effects in CAR-T Therapy

Traditional CAR-T therapy involves the injection of $CD3^+$ CAR-T cells with nonspecific ratios of $CD8^+$ and $CD4^+$ subsets, leading to varying frequencies of $CD8^+$ and $CD4^+$ T cells in all patients. Due to this discrepancy, it is difficult to set a consistent baseline for gauging the effectiveness of CAR-T therapy as well as maximizing the positive effects of CAR-T. In this study, subsets of T cells were tested for their respective antitumor efficacy to determine the best combination of specific T cells to maximize CAR-T efficacy.

In this study, $CD8^+$ and $CD4^+$ were first tested for subset efficacy and then for the combined effect of superior $CD8^+$ and $CD4^+$ subsets on murine B cell lymphomas in a set ratio (Sommermeyer et al., 2016). $CD8^+$ and $CD4^+$ subsets were tested respectively in NOD SCID IL2RgNULL (NSG) mice. The NSG mice engrafted with $CD19^+$ Raji tumors then tested for cytokine release, cytolytic abilities, and survival grafts, with mice grafted with EGFRt-T cells serving as control. The results revealed a hierarchy of subset effector functions. $CD8^+$ T_{CM} and T_N cells showed more cytolytic ability while $CD4^+$ T_N cells had superior cytokine release. Given that $CD8^+$ T_{CM} and $CD4^+$ T_N had superior antitumor responses, doses of either $CD8^+$ T_{CM}, $CD4^+$ T_N, or both of them combined in a 1:1 ratio were administered to the mice. Mice that received a 1:1 of $CD8^+$ T_{CM} and $CD4^+$ T_N showed better survival rates as well as more complete tumor eradication, judging from the bioluminescence imaging data and survival curve. This data leads to the conclusion that certain defined T cell subsets do enhance antitumor response, in this case, a 1:1 combination of $CD8^+$ T_{CM} and $CD4^+$ T_N.

This study is significant because the antitumoral efficacy of CAR-T cell subsets has never been previously studied, and it is revealed that subsets of $CD8^+$ T_{CM} and CD4+ CAR-TN have the strongest antitumoral effects. Moreover, this study demonstrates combining CD8+ TCM and CD4+ TN cells in a 1:1 ratio has a synergistic antitumor effect than exclusively using one subset (Sallusto, 1999).

3 DISCUSSION

Upon further examination of current research studies, we hypothesized a new experimental CAR-T therapy to maximize CAR-T efficacy while reducing side effects. This new CAR-T therapy will incorporate all the modification processes shown in previous research studies, including the self-secreting anti-PD-1 CAR, the ability to secrete CD40 ligands and the IL-6 binding proteins all administered in a 1:1 $CD8^+$ T_{CM} to $CD4^+$ T_N cells (figure 2), which, if proven to be successful, can significantly increase the efficacy of CAR immunotherapy while reducing the potential side effects to the lowest level.

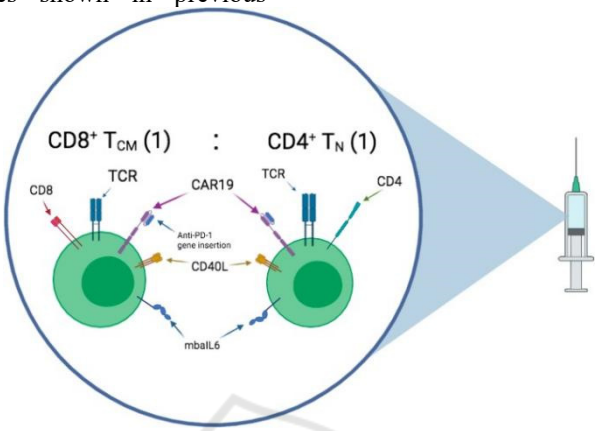

Figure 2: Cell model in the modified CAR-T cancer therapy.

CAR-T cells that target CD19 antigen (CAR19) will be engineered to express anti-PD-1 antibody (Anti-PD-1), CD40 ligand (CD40L), and membrane binding IL-6 receptors (mbaIL6). These new proteins are engineered on both CD8-expressing central memory T cells ($CD8^+$ T_{CM}) and CD4-expressing naive T cells ($CD4^+$ T_N), The ratio of the two types of T cells will be manipulated to reach 1: 1 in this new therapy.

To successfully create this new CAR-T therapy targeting B cell malignancy as well as to test the proper expression of different receptors and their effectiveness, we propose several experiments using different methods and analyzed potential results that could reflect whether this new treatment is feasible for future cancer therapies.

3.1 Gene Insertion During CAR-T Engineering

The insertion of genes of anti-PD-1, CD40L, and mbaIL6 achieved by introducing retroviral vectors into T cells. The retroviral vector can accommodate genes of interest and incorporate its genes into the target cell genes (Hambach, 2020). It has been largely used during CAR-T cell engineering because of its high transfer efficiency as well as its variety of gene expression based on different types of viruses used (Guedan,, 2019). In our proposed experiments on CAR engineering, the retroviral vector of MP71 will be used to insert the ScFv of the anti-PD-1 antibody derived from human mAb 5C4 (Habib, 2019); MSCV vector will be used as the basis to transduce membrane bond anti-IL-6 derived from human anti-IL-6 monoclonal antibody AME-19a (Neelapu, 2017); finally, the SFG-m1928z-CD40L will be constructed using Gibson Assembly (Elgueta, 2009)..

3.2 Testing Successful Expression of Engineered Receptors Using Western Blot

The successful expression of multiple receptors in our proposed CAR-Ts can be detected using western blot, a technique used to identify specific proteins of interest through the binding of specially designed antibodies. After binding to these designed antibodies, proteins of interest will be stained and visualized through gel electrophoresis. Different types of proteins in a protein mixture will be separated based on their molecular size (Kurian, 2020). Based on previous studies, it has been found that CD40L has a molecular weight between 32 to 39 kDa (Odorizzi, 2012), while anti-PD-1-producing CAR has a molecular weight of approximately 27kDa (Habib, 2019). Since these two proteins have a significant weight difference, antibodies designed separately for these two proteins can be added together in western blot using the original CD19 CAR as the control group. If the gel electrophoresis result shows two distinct stains, one located on 27

kDa and the other between the range of 32 to 39 kDa on the newly designed CAR-T cells while no clear stain for the control group, it can be indicated that CD40L and anti-PD-1-producing CAR has been successfully expressed. If no stains are shown, or they indicate different molecular weights, then the engineered protein may not be successfully expressed.

3.3 Detection and Isolation of Target T cells Using Flow Cytometry Assay

In this experiment, we aim to isolate $CD8^+$ central memory T cells and $CD4^+$ naïve T cells based on their surface marker proteins using flow cytometry assay. As naïve T cells predominantly express CD62L (Sallusto, 1999), we designed antibodies specifically targeting CD4 and CD62L. After the flow cytometry assay, cells showing higher affinity to both CD4 and CD62L will be collected and used as the naïve T cell in the therapy. Because central memory cells are have CCR7+, the remaining memory T cells expressing CCR7 will be further isolated using anti-CCR7, completing isolation of CD4+ TN and CD8+ TCM. After extracting T_{CM} and T_N cells, we propose to further isolated T cells that simultaneously express all three receptors engineered on the new CAR-T cell using fluorescence-activated cell sorting (FACS). The target T cells are identified and extracted based on their binding intensity to antibodies designed for the three different proteins. Based on previous research, we have determined it is best to administer the $CD8^+$ T_{CM} cells and $CD4^+$ T_N cells in a 1: 1 ratio, which has shown to have the greatest synergistic antitumor effect (Sallusto, 1999). Therefore, after isolating the target T cell subsets, we will manipulate a 1: 1 $CD8^+$ CAR-T_{CM} to $CD4^+$ CAR-T_N ratio before conducting experiments *in vivo.*

3.4 Testing mbaIL-6's Effective Reduction of CRS

Infusion of CAR-T cells could lead to potentially life-threatening CRS, which would sharply increase the cost related to this treatment. Based on previous experiments of mbaIL6, we decided to insert gene encoding mbaIL6 to our newly designed ultimate CAR-T cells to stay one step ahead of current studies by combining mbaIL6 with other improvements on CAR-T cells. Transduction of T lymphocytes could be done with a construct that allows simultaneous expression of mbaIL6, CAR, anti-PD1, and CD40L to approach more effective antitumor capacity without the presence of CRS. This construct could be placed on an MSCV retroviral vector containing genes of mbaIL6, anti-CD 19 CAR, anti-PD1, and CD40L. For precise detection of anti-CD 19, CD19-myc will be connected to the extracellular domain of the CD19 molecule, and cells are stained with the CD19-myc fusion protein and an anti-myc antibody. Through variable control, degree of expression of mbaIL6, CAR, anti-PD1, and CD40L, cell marker profile (CD4, CD8, etc.), the proportion of naive, effector, central memory, and effector memory, and level of IL-6 Neutralization performed by mbaIL6 can be observed. To test whether IL-6 neutralization by mbaIL6 can interfere with antitumor capacity activated by CAR, we allow T cells to coculture with $CD19^+$ OP-1 ALL cells and measure IFN-γ production by flow cytometry assay after labeling with anti-human IFN-γ–PE. The Level of cytotoxic granules released by ultimate CAR-T cells can be tested by staining with the anti-CD107a antibody. The xenograft models could be used to determine the in vivo antitumor capacity of ultimate CAR-T cells, CD191 ALL cell line Nalm-6 IV (a B cell precursor leukemia cell line) will be injected in NSG mice along with ultimate CAR-T with and without mbaIL6. Furthermore, levels of human IL-6 neutralization in vivo can be determined through injecting ultimate CAR-T with and without mbaIL6 accompanied by IP injection of human IL-6 several days later. If this combination works in xenograft models, we would see considerable antitumor capacity with and without expression of mbaIL6 on ultimate CAR-T for a relatively long period. Significantly, ultimate CAR-T expressing mbaIL6 can neutralize mbaIL6 to a lower level compared with ultimate CAR-T not expressing mbaIL6 and prevent CRS (Neelapu, 2017).

3.5 Testing T Cell Efficacy Through Cytolytic Ability and Survival Rate of Engrafted Mice

After injecting our proposed CAR-T therapy in the NSG mice models, the efficacy of our modified CAR-T therapy, we propose to use chromium release assay as a measurement of cytolytic activity and survival grafts to detect survival rates of mice bearing CD-19 B lymphomas. Mice receiving only EGFRt-T cells will serve as the control. If this combined treatment were effective, we would expect to see a significant increase in the survival rates and cytolytic abilities of mice receiving the modified CAR-T therapy compared to those receiving the control. However, if the chromium release assay and survival

rates show no significant difference or show a significant decrease between the modified CAR-T group and the control, we can deduce that our modified CAR-T therapy fails to have a synergistic effect on tumor masses.

4 CONCLUSION

In this study, we looked into six current research on various CAR cancer therapies. Based on the methods and results shown in these research experiments, proposed a new CAR-T therapy model that combines anti-PD1 and CD40L secretion, mbaIL6 receptor. To further improve the efficacy of this new model, we also suggested an optimal 1:1 CD8 T_{CM} to CD4 T_N cell ratio. The proposed experiment will be tested *in vivo* using the NSG mice model, which, if proven to be successful, can significantly improve the overall CAR-T cell efficacy in treating B cell lymphoma. However, because we are unable to conduct actual experiments, the feasibility and overall efficacy of this newly designed CAR-T therapy will have to be thoroughly investigated in future experiments.

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